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BIOMARKERS OF HEPATIC INJURY AND FUNCTION IN NEONATAL HYPOXIC ISCHEMIC ENCEPHALOPATHY AND WITH THERAPEUTIC HYPOTHERMIA

--Manuscript Draft--

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Abstract:	Therapeutic hypothermia (TH) is now provided as standard care to infants with moderate-severe hypoxic ischemic encephalopathy (HIE). The role of TH in limiting neuronal injury is well recognized, but its effect on hepatic injury which occurs frequently in neonatal HIE is not known. Our objective was to characterize biomarkers of liver injury and function in the setting of neonatal HIE and to describe whether HIE severity and provision of TH influence these hepatic biomarkers. We performed a multicenter retrospective study and compared hepatic biomarkers obtained during the first postnatal week, according to the severity of HIE and whether treated with TH. Of a total of 361 infants with HIE, 223 (62%) received TH and 138 (38%) were managed at normal temperature. Most hepatic biomarkers and C-reactive protein (CRP) were significantly associated with the severity of HIE ($p<0.001$). Infants treated with TH had

	lower peak Alanine aminotransferase (ALT) concentrations ($p=0.025$) and delay in reaching peak CRP concentration ($p<0.001$). Conclusion: We observed a significant association between the clinical grade of HIE and biomarkers of liver metabolism and function. Therapeutic hypothermia was associated with delayed CRP responses and with lower ALT concentrations and so may have the potential to modulate hepatic injury.

Dear Editorial team and Reviewers,

Many thanks for your thorough review of our manuscript titled " Biomarkers of hepatic injury and function in neonatal hypoxic ischemic encephalopathy and with therapeutic hypothermia" and for your valuable suggestions and comments. We have made changes in the revised manuscript based on your comments. Please find below, responses to individual reviewers' comments.

Reviewer #1

1. Though there was no statistical difference in ALT in Normothermia and Hypothermia group (Table3) but after applying Regression coeffficients (table4) there was statistical difference. It is appropriate application of statistical principles in current scenario. It would be helpful to compare the Normothermia groups separately with all three grades of HIE , e.g Normothermia Grade3 HIE with Hypothermia Grade 3 HIE group and others.

   As per reviewer's suggestion, we performed the analysis comparing normothermia and hypothermia groups based on separate grades of HIE and included the analysis as a new table 4 (provided as online resource) in the revised manuscript. Changes were made to methods (line 68-70) and results section (line 112-114) accordingly.

   2. Hypothermia has been shown to be helpful in Moderate and Severe Encephalopathy as authors also mention in Introduction. There were few cases of mild HIE that also received hypthermia. Those values can dilute the accuracy of measurements so It would be great if authors can look at comparison of Liver enzyme levels of only Moderate and severe HIE cases from Hypothermia and Normothermia groups.

   We have now performed univariate analysis comparing normothermia group and hypothermia groups in infants with moderate-severe HIE (grade 2 and 3) only and included the analysis as table 4 online resource in the revised manuscript. Changes were made to methods (line 68-70) and results section (line 115-117) accordingly.

   3. For CRP measurement- Serial trending is most important when assessing information.For Figure 2 and its interpretation, more clarity is needed as to How many CRP measurements were recorded and displayed in the graph? Any cases where there were more than 1 CRP measurements displayed on the graph. If only 1 measurement was selected, which one (day of life) was chosen and why? Did we use only the peak concentration if there were multiple values.

more clarity is needed as to How many CRP measurements were recorded and displayed in the graph?

We used daily mean concentrations of all recorded peak CRP concentrations with standard error of mean in each group (normothermia and hypothermia) to evaluate changes over the first 7 days of life. CRP was recorded in 99% of the infants with median of 5 recordings (1-7) in the first 7 days. Since we wanted to note the trend over the 7 days, we decided to include the mean (SEM) of the individual peak CRP concentrations available each day for the 7 days similar to studies performed by Perrone et al and Chakkarapani et al who evaluated effect of hypothermia on CRP response in infants with HIE. Thus babies with only one CRP available were not excluded from this analysis, irrespective of day of obtainment. In the figure we note that the CRP concentrations (mean) peak is delayed in infants with hypothermia. These findings were consistent when we also looked at days to peak for individual infants with hypothermia in comparison with normothermia group. (Tables 3 and 5).

We have made changes in the result section (line 139-141) to clarify this information.

Did we use only the peak CRP concentration if there were multiple values.

Yes, if there were multiple daily values, the peak CRP was chosen in data collection. We have clarified this in the Fig 2 legend title by addition of word 'Peak'.

Reviewer #2:
1. Does the later increase in CRP in cooled infants reflects the effect of cooling on inflammation ? This could be worked out in the discussion.

We have revised our Discussion section on hypothermia and CRP (line 200-203) in response to the reviewer's helpful comment as below
"Hypothermia is known to modulate leucocyte and immune responses with altered and delayed expression of inflammatory mediators and cytokines including IL-6. [19-21]. CRP is an acute phase reactant protein produced in the liver in response to pro-inflammatory cytokine IL-6.[30]"

Delay in expression of cytokines with hypothermia is likely to delay CRP response in infants treated with therapeutic hypothermia.

2. I believe it is important that both liver problems and HIE are due to peripartal asphyxia and the severity of peripartal asphyxia. In the text, the authors correlate HIE and liver function but it has to be stressed that they are both caused by peripartal asphyxia.

Thank you for the comment. We have made changes to the Introduction (L26) and Discussion section 150-151 to address the above comments.

We have also made a small number of other minor changes/corrections in the revised manuscript, which have been tracked".

I would once again like to thank the editors and reviewers for their comments and suggestions.

I hope you will find the revised manuscript suitable for publication in European journal of Pediatrics.

Kind regards

Hemananda Muniraman

TITLE:

BIOMARKERS OF HEPATIC INJURY AND FUNCTION IN NEONATAL HYPOXIC ISCHEMIC ENCEPHALOPATHY AND WITH THERAPEUTIC HYPOTHERMIA

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ABSTRACT

Therapeutic hypothermia (TH) is now provided as standard care to infants with moderate-severe hypoxic ischemic encephalopathy (HIE). The role of TH in limiting neuronal injury is well recognized, but its effect on hepatic injury which occurs frequently in neonatal HIE is not known. Our objective was to characterize biomarkers of liver injury and function in the setting of neonatal HIE and to describe whether HIE severity and provision of TH influence these hepatic biomarkers. We performed a multicenter retrospective study and compared hepatic biomarkers obtained during the first postnatal week, according to the severity of HIE and whether treated with TH. Of a total of 361 infants with HIE, 223 (62%) received TH and 138 (38%) were managed at normal temperature. Most hepatic biomarkers and C-reactive protein (CRP) were significantly associated with the severity of HIE ($p<0.001$). Infants treated with TH had lower peak Alanine aminotransferase (ALT) concentrations ($p=0.025$) and delay in reaching peak CRP concentration ($p<0.001$).

***Conclusion*:** We observed a significant association between the clinical grade of HIE and biomarkers of liver metabolism and function. Therapeutic hypothermia was associated with delayed CRP responses and with lower ALT concentrations and so may have the potential to modulate hepatic injury.

Key Words: therapeutic hypothermia, liver enzymes, C-reactive protein, perinatal asphyxia, biomarkers

What is known:

- Ischemic hepatic injury occurs frequently as a part of multi-organ dysfunction in infants with hypoxic ischemic encephalopathy (HIE).
- The neuroprotective role of therapeutic hypothermia in management of infants with HIE is well recognized, but the potential hepato-protective effects of hypothermia are unclear.

What is new/What this study adds:

- This large, multi-center study examined commonly-measured biomarkers of hepatic injury and metabolism and showed that therapeutic hypothermia was associated with lower alanine aminotransferase and albumin concentrations and a delayed C-reactive protein (CRP) response.
- An elevated CRP concentration during the first postnatal week may be regarded as an expected finding in moderate and severe HIE, and in the overwhelming majority of cases this appears to occur secondary to hepatic hypoxia-ischemia and in the absence of blood-culture positive sepsis.
- Therapeutic hypothermia may have the potential to modulate hepatic injury.

List of abbreviations: ALB; Albumin, ALT; alanine aminotransferase, AST; aspartate aminotransferase; CB; conjugated bilirubin, CRP; C-reactive protein, GGT; gamma glutaryl transpeptidase, HIE; hypoxic-ischemic encephalopathy, NE; Neonatal Encephalopathy, PTT; Partial thromboplastin time, PT; Prothrombin time, TH; Therapeutic hypothermia.

INTRODUCTION:

Hypoxic-ischemic hepatic injury occurs frequently as a part of multi-organ involvement in neonatal hypoxic ischemic encephalopathy (HIE).[1–3] The pattern of hepatic injury is consistent with the hepatic ischemic insult seen in adults and children following cardiac arrest, namely there is elevation of liver enzymes in the first few days after the insult and normalization within a few weeks.[4–6] Although a few small studies have examined hepatic enzyme changes in the setting of HIE,[6–11] the effects of perinatal asphyxia on hepatic function and recovery are not well characterized, and the value of routine measurement of liver enzymes in infants admitted with suspected HIE is uncertain.

Moderate whole body hypothermia is now provided as standard care to infants with moderate-severe HIE. The benefits of hypothermia in limiting neuronal injury and improving neurodevelopmental outcomes have been well documented[12–15]. While some of the large randomized controlled trials of therapeutic hypothermia included study of liver enzymes elevation as secondary outcomes or reported them as adverse events[14,15], so far no studies have set out primarily to examine the potential influence of therapeutic hypothermia on hepatic biomarkers in infants with HIE.

C-reactive protein (CRP) is an acute phase reactant produced in the liver and is commonly measured in sick neonates in intensive care as a surrogate marker of infection.[16,17] CRP concentrations may also be influenced by perinatal asphyxia in the presence of multi-organ involvement and in the absence of systemic infection. To date only a few reports describe the relationship between perinatal hypoxia-ischemia, therapeutic hypothermia, and CRP responses.[18–22]

Our aims in this study were to: i) characterize hepatic injury in setting of HIE by analyzing the hepatic biochemical markers and CRP concentrations in term and near-term

25 infants during the first postnatal week, and ii) describe any changes in markers of hepatic
26 function and injury associated with severity of HIE and with provision of therapeutic
27 hypothermia.

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METHODS:

This was a retrospective review of clinical records conducted in four tertiary-level neonatal intensive care units (NICUs) in the United Kingdom (UK). Infants eligible for inclusion were those born at ≥36 weeks gestational age in the 5-year period 1st July 2006 to 30th June 2011, and admitted to a participating NICU with a recorded diagnosis at death/discharge of HIE of any clinical severity (grades 1–3 Sarnat-Sarnat). We excluded infants who had a major congenital anomaly or a primary diagnosis of an inborn error of metabolism. Participating centers introduced routine whole-body therapeutic hypothermia for treating HIE at different times within this epoch.

Using a dedicated study proforma**,** we collected the results of all daily blood tests done in eligible infants from admission until completion of 7 postnatal days, or until death if it occurred earlier. We examined the following potential biomarkers of hepatic metabolism: alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma glutamic transpeptidase (GGT), total and conjugated bilirubin, prothrombin time (PT), partial thromboplastin time (PTT) and C-reactive protein (CRP). For each biomarker we recorded the peak plasma concentration each day during the first week, the postnatal day of occurrence of its peak concentration, and the proportions of infants having at least one abnormally elevated value within the first week. We also recorded the nadir plasma albumin concentration, postnatal day of occurrence, and the proportion of infants with an abnormally low albumin level. The following values were considered abnormal and therefore thresholds marking potential liver dysfunction: CRP ≥10 mg/L,[21] ALT >50 U/L, AST >140 U/L, GGT >263 U/L, PT >14.4 s, PTT >51.2 s, and Albumin <26 g/l.[23]. All blood tests had been performed according to routine local clinical practices and at the discretion of the attendant clinicians. We collected baseline data including clinical HIE grade as stated on the discharge summary, timing and duration of any therapeutic hypothermia given, and details of blood

culture results and any associated maternal pyrexia or histopathologically-confirmed chorioamnionitis.

Differences in baseline characteristics of infants with different grades of HIE and between hypothermia and control group were analyzed using the Kruskal-Wallis test, and Mann-Whitney test for non-parametric variables. Chi-squared and Fisher's exact tests were used, as appropriate, to analyze proportions. The association between the potential markers of hepatic dysfunction and severity of HIE was analyzed using the Kruskal-Wallis test. Any biomarkers measured in less than a third of the cohort overall were excluded from analysis.

The effect of hypothermia on the hepatic biomarkers was analyzed by comparing the peak concentrations of the hepatic enzymes and CRP between the cohort of neonates who received whole body therapeutic hypothermia ('hypothermia group') and the cohort who did not receive therapeutic hypothermia ('normothermia group') using the Mann-Whitney test. As both hypothermia and normothermia groups included infants with varying severity of HIE, infants were also stratified based on grade of HIE and an additional analysis was made limited to those with moderate or severe HIE (grade 2 and 3 combined) after excluding mild (grade 1) cases. Furthermore, a multiple regression analysis using logarithmically-transformed values for each hepatic biomarker was performed for the complete cohort to assess the association between extreme values of each analyte and the reception of therapeutic hypothermia after adjusting for grade of HIE and birth weight.

This study had prior approval from a UK National Research Ethics Service ethics committee (REC reference: 11/EE/0349).

RESULTS:

361 eligible neonates were admitted to the four centers during the study period: 101 (28%) had grade 1 HIE, 165 (46%) had grade 2 HIE, and 95 (26%) had grade 3 HIE. In total, 138 (38%) infants were managed at normal temperature (n= 69 HIE grade 1; n=47 HIE grade 2; n=22 HIE grade 3), and 223 (62%) received therapeutic hypothermia (n=32 HIE grade 1; n=118 HIE grade 2; and n=73 HIE grade 3).

For each hepatic biomarker, the percentage (and number) of eligible infants having at least one recorded sample available in the first 7 days, along with median (range) number of samples were as follows: ALT 96% (347/361), 3 (1-7); Albumin 99% (356/361), 5 (1-7); CRP 99% 357/361, 5 (1-7); Total Bilirubin 81% (294/361), 5(1-7); PT 77% (278/361), 1(1-7); PTT 64% (238/361),1(1-7) and AST 40% (145/361) 3(1-6). Conjugated bilirubin and GGT were excluded from analysis because values for these were available in only 22% (80/361) and 20% (71/361) cases respectively.

Baseline patient characteristics are shown in Table 1, with comparison according to HIE grade and reception of hypothermia. The overall rate of culture-positive infection among the whole cohort was 2.8% (10/361) with no difference between the HIE grades or between hypothermia and normothermia groups. The results of histopathological placental examination were available for only 57 (15.7%) of the 361 infants, of which 18 (32%) showed evidence of chorioaminonitis and/or funisitis.[24]

Effects of HIE severity and therapeutic hypothermia on hepatic biomarkers

Table 2 presents the peak values of hepatic biomarkers measured in the first postnatal week, and the proportions of infants having a raised value for each biomarker according to HIE grade. The peak values of the hepatic biomarkers of injury including ALT and AST increased

with severity of HIE grade ($p<0.001$). Similarly, higher proportions of infants were affected with abnormally elevated ALT and AST concentrations with increasing HIE severity.

The biomarkers reflecting hepatic synthetic function, namely albumin and PT, differed according to HIE grade: infants with more severe HIE had significantly lower nadir albumin concentrations and lower peak total bilirubin concentrations (both $p<0.0001$), and a longer PT ($p<0.0001$), Table 2. Proportions of infants affected by an abnormally low plasma albumin value and a prolonged PT were also higher with increasing HIE severity (Table 2, figure 1).

Table 3 shows the results of univariate analysis according to reception of hypothermia treatment. Comparison between the hypothermia-treated and normothermia groups showed lower nadir albumin concentrations and longer PT and PTT times with hypothermia, but no differences for the other hepatic biomarkers. Sub-grouping according to grade of HIE showed significant differences associated with hypothermia reception for only a lower nadir albumin in grade 1 HIE and a longer PTT in grade 2 HIE (Online Resource Table 4). Univariate analysis limited to the sub-group of infants with moderate or severe encephalopathies (grades 2 and 3 HIE combined) showed only a longer PTT was associated with hypothermia therapy (Online Resource Table 4).

After adjusting for grade of HIE and birth weight in a multivariate regression analysis, only ALT and albumin were significantly affected by therapeutic hypothermia: infants in the hypothermia group had lower peak ALT ($p=0.025$) and a lower nadir plasma albumin ($p=0.049$) compared with the normothermia infants, and there were no differences between the hypothermia and normothermia-treated infants for any of the other biomarkers including AST, bilirubin, PT, and PTT (Table 5).

Effect of HIE severity and therapeutic hypothermia on peak CRP concentration

A raised CRP was present in 206/357 (57.7%) neonates during the first postnatal week (Table 2), with the peak occurring on postnatal day 4 overall. Proportions with a raised CRP increased with severity of HIE grade ($p<0.0001$). Considering only neonates with moderate or severe HIE (grades 2 and 3), it is noteworthy that the majority had a raised CRP within the first postnatal week (166/258; 64.3%), while only a small minority (8/246; 3.3%) had culture-positive sepsis (Table 1). CRP concentrations also peaked later in grades 2 and 3 HIE compared to grade 1 HIE ($p=0.0001$) (Table 2).

Univariate analysis showed that compared with HIE infants who did not receive hypothermia, the hypothermia-treated group had a higher peak CRP (15.4 versus 9.3 mg/L, $p=0.01$) and a higher proportion of infants with a raised CRP (62.6% versus 49.6%) (Table 3). After adjusting for HIE grade and birth weight, the multivariate regression showed no difference in peak CRP concentration between hypothermia and normothermia groups ($p=0.5$) however the time to peak was delayed in the hypothermia-treated group ($p<0.001$) (Table 5).

Figure 2 depicts changes in daily mean concentrations of peak CRP values over the first 7 days of life in normothermia and hypothermia groups with a delayed peak noted in infants who received hypothermia.

DISCUSSION:

With this study, we sought to determine the effect of hypoxic injury on surrogate biomarkers of hepatocellular integrity (ALT, AST) and hepatic synthetic function (albumin, PT) in neonates with HIE.[25] We believe this is the largest study to characterize markers of hepatic injury and function in setting of neonatal HIE [6-11] and, to our knowledge, the first to present baseline reference values for a range of hepatic biomarkers in the era of routine therapeutic hypothermia. Both hypoxic-ischemic neuronal and hepatic injury can occur secondary to perinatal asphyxia. We observed significant correlations between severity of HIE and values of several hepatic biochemical markers within the first 7 days after birth. More severe HIE was associated with greater elevation of hepatic enzymes (ALT, AST) and with abnormalities of markers of hepatic synthetic function (Albumin, PT).

Effect of severity of HIE on hepatic biochemical markers

Several smaller studies have reported an increase in some hepatic enzymes in infants with perinatal asphyxia and neonatal encephalopathy, including for AST and ALT.[3,6-10] Some have examined the correlation of hepatic biomarkers with severity of encephalopathy.[6,8-10] Of these, three reported significant correlation between hepatic enzymes and severity of neonatal encephalopathy,[6,8,10] while one study reported no correlation.[9] The inconsistency may be due to the relatively small numbers of infants studied, differing definitions of abnormal values of hepatic markers, and small cohorts making them relatively under powered for assessing correlations with HIE severity.

Changes in hepatic biomarkers with therapeutic hypothermia

Hypothermia limits neuronal injury in neonates with HIE,[26, 27] and improves neuro-developmental outcomes,[12-15] however effects of hypothermia on other organ systems are

less well studied. Vejchapipat et al. performed an experimental study using a rat model and reported that moderate hypothermia (30-33°C) ameliorates liver energy failure compared to controls after intestinal ischemic reperfusion injury.[28]. A meta-analysis of six randomized controlled trials which included 975 infants (316 of whom had hepatic dysfunction defined by using a higher threshold of AST >200 U/L and/or ALT >100 U/L), showed no significant hepato-protective effect of therapeutic hypothermia (relative risk 0.88 [95% CI: 0.74 to 1.05]).[29] However, the frequency and completeness of liver function testing in neonates in the included trials was unclear, and the use of a stricter definition of liver dysfunction may have decreased the sensitivity for detecting an effect. In our cohort, we observed inconsistent results for individual hepatic markers, with significantly lower peak ALT concentrations in the hypothermia group, but no difference for AST concentrations. This may possibly be due to the relatively lower number of babies with available AST samples. Nevertheless, ALT is considered to be a more specific marker for hepatic injury than AST which can be elevated due to other non-hepatic causes.[7,25] The biomarkers of hepatic function again showed varying results with a marginally lower albumin in the hypothermia group ($p=0.049$), but no difference in PT. The latter result is consistent with the meta-analysis of randomized controlled studies of therapeutic hypothermia which found no difference in coagulopathy between the hypothermia and control groups.[29]

Effect of severity of HIE and hypothermia on CRP responses

Several studies have examined CRP concentrations in the setting of HIE.[17-22] Shang et al. found a higher CRP concentration correlated with increasing clinical HIE severity in 74 infants.[18] Our study in a much larger cohort confirms that peak CRP concentrations and also proportions affected by a raised CRP both correlate strongly with HIE severity. Indeed a raised CRP appears to be an expected finding during the first postnatal week in neonates admitted with moderate or severe HIE; in our cohort this was nearly always in the absence of

infection because the rate of culture-positive infection was only 2.8% overall. This discrepancy may have clinical implications because it may help influence a more judicious use of antibiotics in infants admitted with HIE, particularly in those with negative cultures, and perhaps a higher threshold for performing repeated full infection screens later in the first week in the presence of a raised CRP despite invariable initial antibiotic treatment. Despite the low rate of proven sepsis, we found a high rate of chorioamnionitis (32%) for those infants whose placentas had been submitted for examination, highlighting the importance of formal routine placental examination in infants admitted after perinatal asphyxia.[24]

Hypothermia is known to modulate leucocyte and immune responses with altered and delayed expression of inflammatory mediators and cytokines including IL-6. [19-21]. CRP is an acute phase reactant protein produced in the liver in response to the pro-inflammatory cytokine IL-6.[30] Perrone et al. and Chakkarapani et al. compared neonates with encephalopathy who received therapeutic hypothermia with controls who were not treated with hypothermia and also reported a delayed CRP response in hypothermia-treated infants.[20, 21] Okumus et al. recently showed that CRP responses were altered with therapeutic hypothermia with significantly higher levels of CRP, which peaked at day 4 of life compared to a normothermia group which showed no variation in CRP with time.[22] While we did not find any difference in peak CRP levels between our hypothermia-treated infants and those managed at normal temperature, we nevertheless also found a delay in peak CRP responses with therapeutic hypothermia, in line with these previous studies.[20-21]

Our study has a few limitations. This was a retrospective study and hence data were not available for all desired variables. Not all NICUs measured all hepatic biomarkers routinely and consistently, therefore we needed to exclude the biomarkers GGT and conjugated bilirubin where a high proportion of biomarkers were unmeasured. Furthermore, we recognize that not all measured hepatic enzymes and biomarkers are wholly specific for

the liver, for example AST and PT can be elevated due to non hepatic causes. Similarly CRP is commonly elevated in infection and in other inflammatory conditions, although rates of culture-positive sepsis were very low in our cohort. Therapeutic hypothermia was introduced at different intervals in our participating centers during the study period and the normothermia group included infants with moderate and severe HIE, who may have qualified for therapeutic hypothermia before it became standard care. To address this limitation, we performed multiple regression analysis to adjust for effect of severity of HIE on hepatic biomarkers whilst assessing for influence of therapeutic hypothermia. Our analysis of biomarkers was confined to samples obtained in the first postnatal week, and more longitudinal variation in these biomarkers remains unknown. Strengths of our study are that it presents data on hepatic markers associated with HIE in the largest cohort to date, and that it provides preliminary reference ranges for a number of hepatic biomarkers in encephalopathic infants, most of whom received therapeutic hypothermia.

Conclusion:

In our retrospective study of a large cohort of infants with HIE, we have observed a significant association between the clinical grade of HIE and several markers of liver metabolism and function. Therapeutic hypothermia was associated with delayed CRP responses and with lower ALT and albumin concentrations. More studies will be required to prove a definitive effect of hypothermia on limiting hepatic injury. However, as hypothermia is now standard treatment in moderate-severe HIE, future prospective controlled studies will not be possible in human infants and the best inferences may therefore need to come from animal models.

Compliance with Ethical Standards:

Conflict of interest statement: There are no competing interests and no conflicts of interests to declare in relation to this work.

Funding: No specific funding was received for this study.

Ethical approval: This article does not contain any studies with human participants or animals performed by any of the authors and informed consent was not required for the purpose of the study. This study was done with the approval of the National Research Ethics Service Committee East of England - Cambridge Central (REC reference: 11/EE/0349).

Author contributions :

Paul Clarke conceived the idea for this study. Hemananda Muniraman and Paul Clarke designed the study protocol, drafted the data collection form, and obtained the ethics approval. Hemananda Muniraman, Paul Clarke, Sunil Sanka, Danielle Gardner, Anna Paweletz, Anitha Vayalakkad, Ying Hui Chee, Clare Clifford, and Vidheya Venkatesh collected the data from the four centers. Data were analyzed by Jane Skinner, Hemananda Muniraman and Paul Clarke. Anna Curley, Suresh Victor, Mark Turner, and Paul Clarke obtained local approvals for their centers, verified data queries, and provided intellectual input. Hemananda Muniraman and Paul Clarke wrote the first manuscript draft. All authors contributed to manuscript drafting and approve the final version. Paul Clarke is guarantor.

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Figure legends:

Table 1. Baseline characteristics of the 361 infants admitted with hypoxic-ischemic encephalopathy with subdivision according to encephalopathy grade and reception of hypothermia

Table 2. Concentrations of hepatic enzymes and hepatic biomarkers measured in the first postnatal week, with subdivision according to grade of hypoxic ischemic encephalopathy

Table 3. Concentrations of hepatic enzymes and hepatic biomarkers measured in the first postnatal week in normothermia and therapeutic hypothermia groups.

Table 4. (Online Resource) Concentrations of hepatic enzymes and hepatic biomarkers measured in the first postnatal week in normothermia and therapeutic hypothermia groups based on grade of HIE.

Table 5. Regression coefficients of log (biomarkers) with therapeutic hypothermia

Figure 1. Hepatic biomarkers and grades of HIE: The biomarkers are reported in median with interquartile ranges. All infants with at least one measurement available were included.

Figure 2. Peak CRP levels (means with standard error) in the first 7 days of life in therapeutic hypothermia and normothermia groups. All infants with at least one CRP measurement available were included.

Table 1 Hepatic biomarkers

1 Table 1. Baseline characteristics of 361 infants admitted with hypoxic-ischemic encephalopathy with subdivision according to

2 encephalopathy grade and reception of hypothermia

3

	HIE Grade 1 N=101	**HIE Grade 2 N=165**	**HIE Grade 3 N=95**	**P-value**	**Normothermia N=138**	**Hypothermia N=223**	**P-value**
Gestational age, weeks^{+days}	40+2 (36^{+0} to 42^{+3})	40+0 (36^{+0} to 43^{+0})	40+3 (36^{+0} to 42^{+1})	0.122*	40+0 (36^{+0} to 43^{+0})	40+1 (36^{+0} to 42^{+4})	0.934†
Birth weight, g	3460 (2024 to 5400)	3394 (1940 to 5200)	3450 (1450 to 5160)	0.73*	3327 (1940 to 5400)	3500 (1450 to 5200)	0.018†
Male sex, n (%)	62 (61.3)	108 (65.5)	45(47.4)	0.015#	81 (58.7)	134 (60.1)	0.793#
Apgar score at 10 min	7 (2-10)	6 (0-10)	3 (0-9)	<0.0001*	7 (0-10)	5 (0-10)	<0.0001†
Arterial cord pH	7.0(6.79 to 7.32)	6.98 (6.41 to 7.37)	6.90 (6.44 to 7.33)	0.001*	7.03 (6.56 to 7.33)	6.90 (6.41 to 7.37)	<0.001†
First gas pH	7.13 (6.64 to 7.36)	7.01 (6.57 to 7.41)	6.82 (6.40 to 7.35)	<0.0001*	7.07 (6.45 to 7.36)	6.98 (6.4 to 7.41)	0.005†

Cord gas base deficit	–12.0 (–3.6 to –24.5)	–13.8 (–0.5 to –31.4)	–18.8 (–1.9 to –34.1)	<0.0002*	–12.0 (–2.8 to –24.7)	–16.2 (–0.5 to –34.1)	0.001†
Lactate (cord blood or admission), mmol/L	10.8 (2.1 to 26.3)	14.3 (2.6 to 30.8)	18.5 (7.9 to 28.0)	<0.0001*	11.0 (2.1 to 26.7)	14.7 (2.6 to 30.8)	0.0001*
Received hypothermia, n (%)	32 (31.7)	118 (71.5)	73 (76.8)	<0.0001#	0(0)	223 (100)	N/A
Maternal pyrexia, n (%)	6 (5.9)	7 (4.2)	1 (1.1)	0.19#	7 (5.1)	7 (3.1)	0.36#
Culture positive sepsis¶, n (%)	2/95 (2.1)	6/154 (3.9)	2/92 (2.2)	0.77#	5/124 (4.2)	5/217 (2.3)	0.36#
Survival to discharge, n (%)	101 (100)	161 (97.6)	56 (58.9)	<0.0001#	126 (91.3)	192 (86.0)	0.14#

Data are medians with ranges unless indicated.

*Kruskal-Wallis test, †Mann-Whitney test, #Chi-squared or Fisher's exact test.

¶Reported for cases where blood cultures were done

Table 2: Concentrations of hepatic enzymes and hepatic biomarkers measured in the first postnatal week, with subdivision according to clinical grade of hypoxic ischemic encephalopathy

	HIE grade 1 N=101	**HIE grade 2 N=165**	**HIE grade 3 N=95**	**HIE All grades N=361**	**P-value***
ALT	***n = 93***	***n = 162***	***n = 92***	***n = 347***	
Maximum ALT, U/L	54 (8 to 656)	90 (10 to 1796)	149 (3 to 1903)	89 (3 to 1903)	<0.001
No. with elevated ALT (>50 U/L), n (%)	47 (50.5)	105 (64.8)	71 (77.2)	223 (64.3)	<0.001
Postnatal day of peak ALT	2 (1 to 7)	2 (1 to 7)	2 (1 to 7)	2 (1 to 7)	0.37
AST	***n = 35***	***n = 81***	***n = 29***	***n = 145***	
Maximum AST, U/L	118 (36 to 855)	212 (18 to 4728)	465 (68 to 3150)	209 (18-4728)	<0.001
No. with elevated AST (>140 U/L), n (%)	14 (40.0)	56 (69.1)	24 (82.8)	94 (64.3)	<0.001
Postnatal day of peak AST	1 (1 to 2)	1 (1 to 4)	1 (1 to 4)	1 (1 to 4)	0.76
PT	***n = 57***	***n = 136***	***n = 85***	***n = 278***	
Maximum PT, s	16.1 (9.3 to 54.4)	17.9 (12.0 to 171.0)	22.0 (11.4 to 240.0)	18.0 (9.3 to 240.0)	<0.0001
No. with elevated PT (>14.4 s), n (%)	42 (73.6)	115 (84.6)	78 (91.8)	235 (84.5)	<0.001
Postnatal day of longest PT	1 (1 to 4)	1 (1 to 7)	1 (1 to 5)	1 (1 to 7)	0.16
PTT	***n = 49***	***n = 116***	***n = 73***	***n = 238***	
Maximum PTT, s	37.7 (24.3 to 240.0)	41.0 (21.9 to 240.0)	50.0 (26.5 to 240.0)	41.2 (21.9 to 240.0)	<0.0001
No. with elevated PTT (>51.2 s), n (%)	5 (10.2)	29 (25.0)	33 (45.2)	67 (28.2)	<0.0001
Postnatal day of longest PTT	2 (1 to 3)	1 (1 to 4)	1 (1 to 4)	1 (1 to 4)	0.91

ALBUMIN	***n = 98***	***n = 164***	***n = 94***	***n = 356***	
Lowest ALB g/L	29 (12 to 39)	24 (11 to 37)	20 (7 to 37)	24 (7 to 39)	<0.0001
No. with low ALB (<26 g/L), n (%)	25 (25.5)	111 (67.7)	82 (87.2)	218 (61.2)	<0.0001
Postnatal day of nadir ALB†	3 (1 to 7)	4 (1 to 7)	4 (1 to 7)	4 (1 to 7)	0.98
Total BILIRUBIN	***n = 78***	***n = 143***	***n = 73***	***n = 294***	
Maximum BILI, µmol/L	116 (18 to 376)	110 (24 to 349)	75 (13 to 238)	104 (13 to 376)	<0.0001
Postnatal day of peak BILI in first week	3 (1 to 7)	3 (1 to 7)	2 (1 to 7)	3 (1 to 7)	0.018
CRP	***n = 99***	***n = 164***	***n = 94***	***n = 357***	
Maximum CRP overall, mg/L	8.0 (0.5 to 188.1)	16.0 (1.5 to 305.9)	16.4 (0.5 to 346.5)	13.0 (0.5 to 346.5)	<0.0001
No. with elevated CRP (≥10 mg/L), n (%)	40 (40.4)	101 (61.6)	65 (69.1)	206 (57.7)	0.0001
Postnatal day CRP peaked in first week‡	3 (1 to 7)	4 (2 to 7)	4 (2 to 7)	4 (1 to 7)	<0.0001

Numbers presented in italic for each enzyme/biomarker assay refer to number of individual babies included in analysis with at least one recorded value during the first week.

Data are shown as median (range) unless indicated.

*Kruskal-Wallis test used for comparison of biomarker concentrations between the three HIE grades; Chi-square test used to assess proportions.

† reported only for infants with low albumin value (<26 g/L)

‡ reported only for infants with raised CRP value (>10 mg/L)

ALT, alanine transaminase; AST, aspartate aminotransferase; PT, prothrombin time; PTT, partial thromboplastin time; ALB, albumin; Bili, bilirubin; CRP, C reactive protein.

Table 3: Concentrations of hepatic enzymes and hepatic biomarkers measured in the first postnatal week in normothermia and hypothermia groups

	Normothermia N=138	**Hypothermia N=223**	**P-value***
ALT	***n = 130***	***n = 217***	
Maximum ALT, U/L	107 (3 to 1903)	87.0 (10 to 1491)	0.94
No. with elevated ALT (>50 U/L), n (%)	85 (65.4)	138 (63.6)	0.74
Postnatal day of peak ALT	2 (1 to 5)	2 (1 to 7)	0.02
AST	***n = 38***	***n = 107***	
Maximum AST , U/L	189.0 (36 to 855)	212.0 (18 to 4728)	0.35
No. with elevated AST (>140 U/L), n (%)	20 (52.6)	74 (69.1)	0.67
Postnatal day of peak AST	1 (1 to 4)	1 (1 to 4)	0.09
PT	***n = 89***	***n = 189***	
Maximum PT, s	16.9 (9.3 to 240)	18.3 (11.6 to 180)	0.04
No. with elevated (>14.4 s), n (%)	71 (79.8)	164 (86.8)	0.13
Postnatal day of longest PT	2 (1 to 7)	1 (1 to 4)	<0.0001
PTT	***n = 81***	***n = 157***	
Maximum PTT, s	37.2 (21.9-240)	44.0 (24.3-240)	<0.0001
No. with elevated PTT (>51.2 s), n (%)	16 (19.8)	51 (32.5)	0.04
Postnatal day of longest PTT	2 (1 to 4	1 (1 to 4)	0.37
ALBUMIN	***n = 135***	***n = 221***	
Lowest ALB, g/L	27.0 (11 to 39)	23.0 (7 to 37)	<0.0001
No. with low ALB (<26 g/L), n (%)	59 (43.7)	159 (71.9)	<0.0001
Postnatal day of nadir ALB†	3 (1 to 7)	4 (1 to 7)	0.06
BILIRUBIN	***n = 87***	***n = 207***	
Maximum BILI, µmol/L	114.0 (20 to 376)	100.0 (13 to 349)	0.09
Postnatal day of peak BILI in first week	3 (1 to 7)	3 (1 to 7)	0.21
CRP	***n = 135***	***n = 222***	
Maximum CRP overall, mg/L	9.3 (1 to 230)	15.4 (0.5 to 346.5)	0.01
First available CRP, mg/L	3.0	2.0	0.19
No. with elevated CRP (≥10 mg/L), n (%)	67 (49.6)	139 (62.6)	0.02
Postnatal day CRP peaked in first week‡	3 (1 to 7)	4 (1 to 7)	<0.0001

Numbers presented for biomarker assays refer to number of individual babies included with at least one recorded value during the first week.

Data are shown as median (range) unless indicated
* Mann-Whitney test, except for variables reported as n %, which were chi-squared tests
† reported only for infants with low albumin value (<26 g/L)
ǂ reported only for infants with raised CRP value (>10 mg/L)
ALT, alanine transaminase; AST, aspartate aminotransferase; PT, prothrombin time;
PTT, partial thromboplastin time; ALB, albumin; Bili, bilirubin; CRP, C-reactive protein

Table 5: Regression coefficients of log (biomarkers)[†], with therapeutic hypothermia[‡]

Biomarker	Regression coefficient for TH	95% CI	P-Value
Peak ALT	–0.322	–0.604 to –0.041	0.025
Peak AST	–0.229	–0.624 to 0.165	0.252
Peak PT	–0.006	–0.117 to 0.129	0.926
Peak PTT	0.110	–0.007 to 0.228	0.066
Minimum ALB	–1.168	–2.333 to –0.007	0.049
Peak Bilirubin	–0.044	–0.222 to 0.134	0.627
Peak CRP	0.090	–0.215 to 0.395	0.562
Days to peak CRP	0.884	0.434 to 1.335	<0.001

[†] *Minimum ALB* and *Days to peak CRP* not log transformed.

[‡] Also adjusted for grade of HIE and birth weight.

ALT, alanine transaminase; AST, aspartate aminotransferase; PT, prothrombin time; PTT, partial thromboplastin time; ALB, albumin; Bili, bilirubin; CRP, C-reactive protein; TH, Therapeutic hypothermia

Figure 1 Hepatic biomarkers

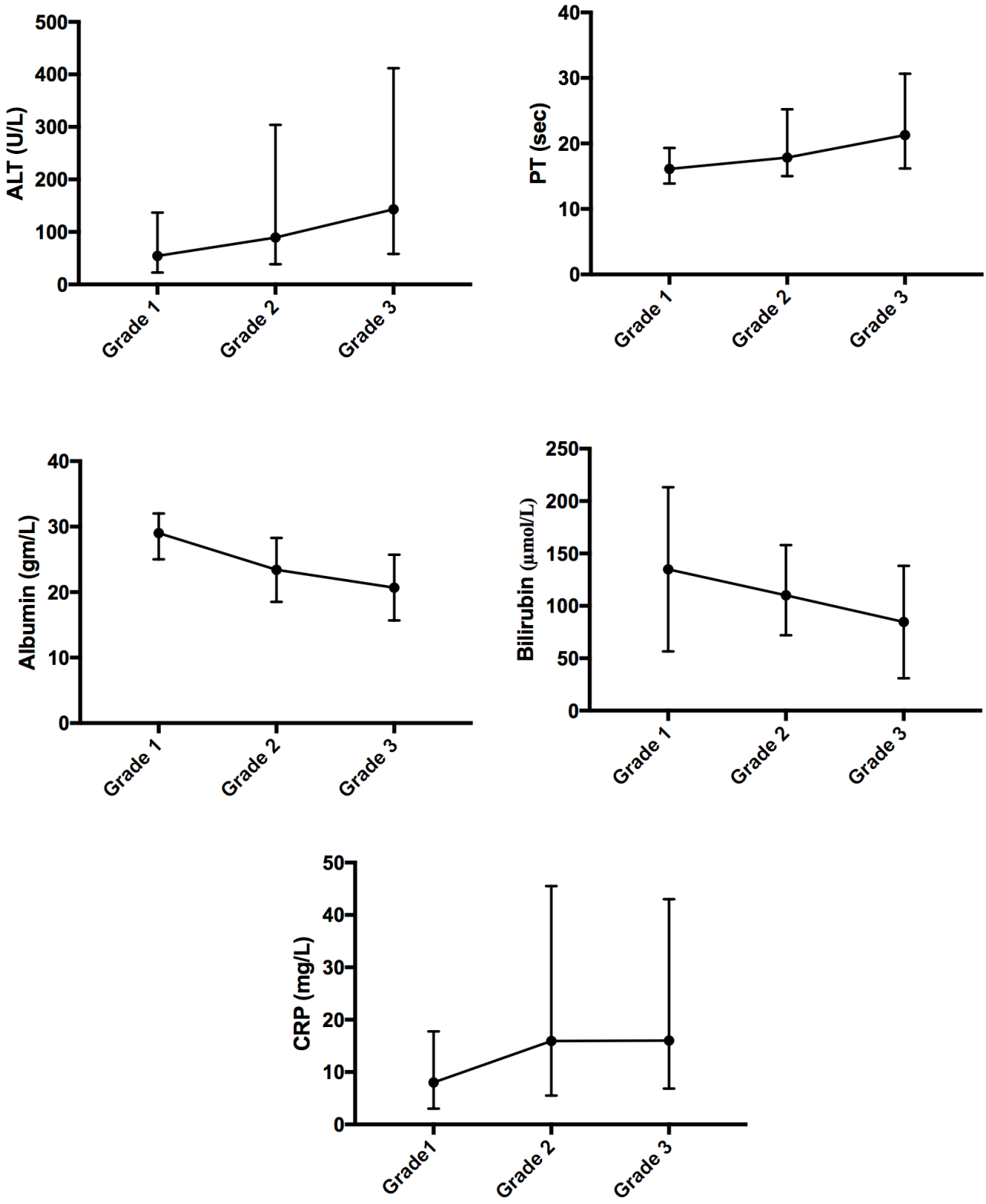

Figure 1: Hepatic biomarkers and grades of HIE: The biomarkers are reported in median with interquartile ranges. All infants with at least one measurement available were included.

Figure 2 Hepatic biomarkers

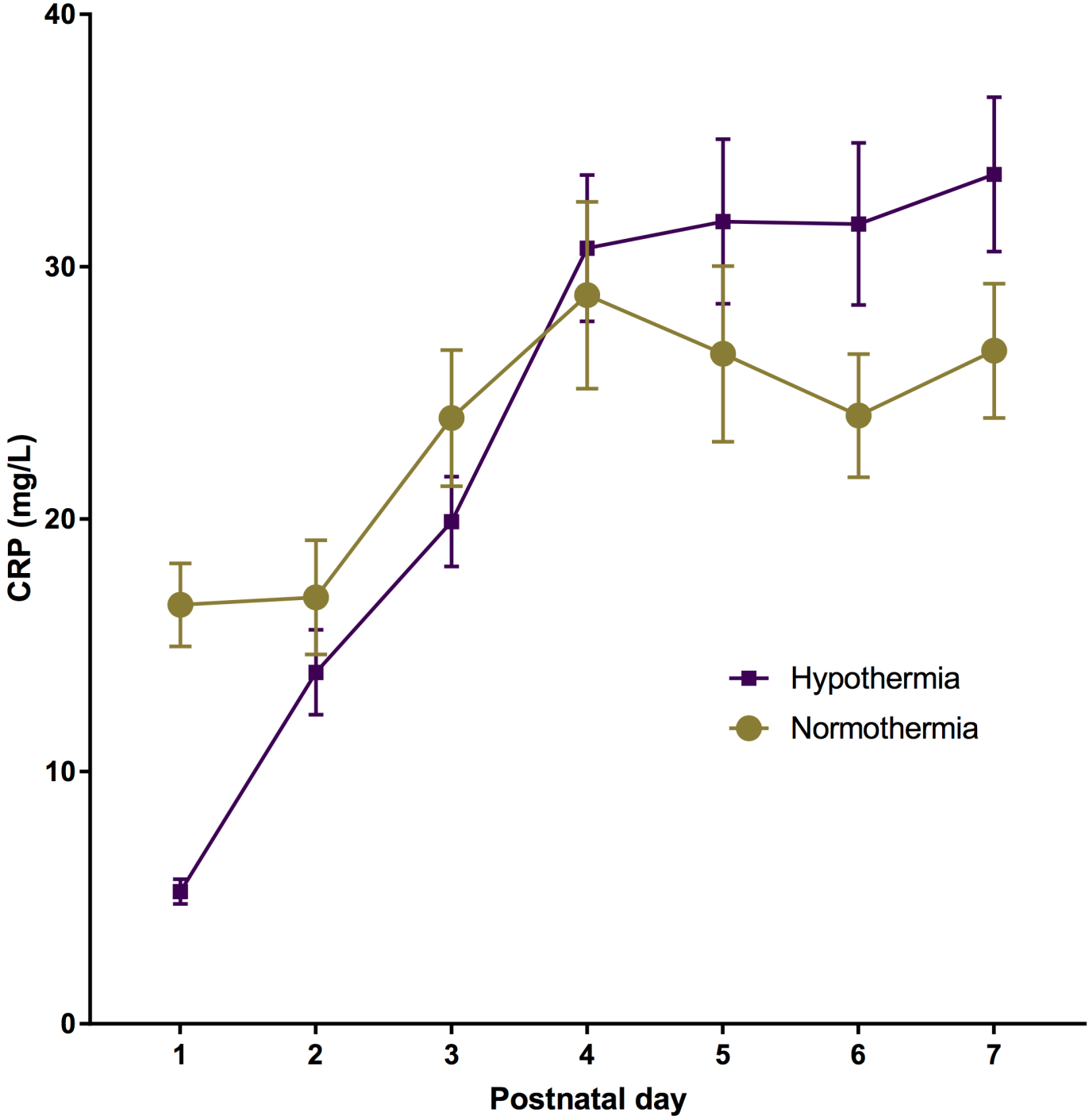

Figure 2: Peak CRP concentrations (mean with standard error) in the first 7 days of life in hypothermia and normothermia groups. All infants with at least one CRP measurement available are included.

INTRODUCTION:

Hypoxic-ischemic hepatic injury occurs frequently as a part of multi-organ involvement in neonatal hypoxic ischemic encephalopathy (HIE).[1–3] The pattern of hepatic injury is consistent with the hepatic ischemic insult seen in adults and children following cardiac arrest, namely there is elevation of liver enzymes in the first few days after the insult and normalization within a few weeks.[4–6] Although a few small studies have examined hepatic enzyme changes in the setting of HIE,[6–11] the effects of perinatal asphyxia on hepatic function and recovery are not well characterized, and the value of routine measurement of liver enzymes in infants admitted with suspected HIE is uncertain.

Moderate whole body hypothermia is now provided as standard care to infants with moderate-severe HIE. The benefits of hypothermia in limiting neuronal injury and improving neurodevelopmental outcomes have been well documented[12–15]. While some of the large randomized controlled trials of therapeutic hypothermia included study of liver enzymes elevation as secondary outcomes or reported them as adverse events[14,15], so far no studies have set out primarily to examine the potential influence of therapeutic hypothermia on hepatic biomarkers in infants with HIE.

C-reactive protein (CRP) is an acute phase reactant produced in the liver and is commonly measured in sick neonates in intensive care as a surrogate marker of infection.[16,17] CRP concentrations may also be influenced by perinatal asphyxia in the presence of multi-organ involvement and in the absence of systemic infection. To date only a few reports describe the relationship between perinatal hypoxia-ischemia, therapeutic hypothermia, and CRP responses.[18–22]

Our aims in this study were to: i) characterize hepatic injury in setting of HIE by analyzing the hepatic biochemical markers and CRP concentrations in term and near-term

infants during the first postnatal week, and ii) describe any changes in markers of hepatic function and injury associated with severity of HIE and with provision of therapeutic hypothermia.

METHODS:

This was a retrospective review of clinical records conducted in four tertiary-level neonatal intensive care units (NICUs) in the United Kingdom (UK). Infants eligible for inclusion were those born at ≥36 weeks gestational age in the 5-year period 1st July 2006 to 30th June 2011, and admitted to a participating NICU with a recorded diagnosis at death/discharge of HIE of any clinical severity (grades 1–3 Sarnat-Sarnat). We excluded infants who had a major congenital anomaly or a primary diagnosis of an inborn error of metabolism. Participating centers introduced routine whole-body therapeutic hypothermia for treating HIE at different times within this epoch.

Using a dedicated study proforma**,** we collected the results of all daily blood tests done in eligible infants from admission until completion of 7 postnatal days, or until death if it occurred earlier. We examined the following potential biomarkers of hepatic metabolism: alanine aminotransferase (ALT), aspartate aminotransferase (AST), gamma glutamic transpeptidase (GGT), total and conjugated bilirubin, prothrombin time (PT), partial thromboplastin time (PTT) and C-reactive protein (CRP). For each biomarker we recorded the peak plasma concentration each day during the first week, the postnatal day of occurrence of its peak concentration, and the proportions of infants having at least one abnormally elevated value within the first week. We also recorded the nadir plasma albumin concentration, postnatal day of occurrence, and the proportion of infants with an abnormally low albumin level. The following values were considered abnormal and therefore thresholds marking potential liver dysfunction: CRP ≥10 mg/L,[21] ALT >50 U/L, AST >140 U/L, GGT >263 U/L, PT >14.4 s, PTT >51.2 s, and Albumin <26 g/l.[23]. All blood tests had been performed according to routine local clinical practices and at the discretion of the attendant clinicians. We collected baseline data including clinical HIE grade as stated on the discharge summary, timing and duration of any therapeutic hypothermia given, and details of blood

culture results and any associated maternal pyrexia or histopathologically-confirmed chorioamnionitis.

Differences in baseline characteristics of infants with different grades of HIE and between hypothermia and control group were analyzed using the Kruskal-Wallis test, and Mann-Whitney test for non-parametric variables. Chi-squared and Fisher's exact tests were used, as appropriate, to analyze proportions. The association between the potential markers of hepatic dysfunction and severity of HIE was analyzed using the Kruskal-Wallis test. Any biomarkers measured in less than a third of the cohort overall were excluded from analysis.

The effect of hypothermia on the hepatic biomarkers was analyzed by comparing the peak concentrations of the hepatic enzymes and CRP between the cohort of neonates who received whole body therapeutic hypothermia ('hypothermia group') and the cohort who did not receive therapeutic hypothermia ('normothermia group') using the Mann-Whitney test. As both hypothermia and normothermia groups included infants with varying severity of HIE, infants were also stratified based on grade of HIE and an additional analysis was made limited to those with moderate or severe HIE (grade 2 and 3 combined) after excluding mild (grade 1) cases. Furthermore, a multiple regression analysis using logarithmically-transformed values for each hepatic biomarker was performed for the complete cohort to assess the association between extreme values of each analyte and the reception of therapeutic hypothermia after adjusting for grade of HIE and birth weight.

This study had prior approval from a UK National Research Ethics Service ethics committee (REC reference: 11/EE/0349).

RESULTS:

361 eligible neonates were admitted to the four centers during the study period: 101 (28%) had grade 1 HIE, 165 (46%) had grade 2 HIE, and 95 (26%) had grade 3 HIE. In total, 138 (38%) infants were managed at normal temperature (n= 69 HIE grade 1; n=47 HIE grade 2; n=22 HIE grade 3), and 223 (62%) received therapeutic hypothermia (n=32 HIE grade 1; n=118 HIE grade 2; and n=73 HIE grade 3).

For each hepatic biomarker, the percentage (and number) of eligible infants having at least one recorded sample available in the first 7 days, along with median (range) number of samples were as follows: ALT 96% (347/361), 3 (1-7); Albumin 99% (356/361), 5 (1-7); CRP 99% 357/361, 5 (1-7); Total Bilirubin 81% (294/361), 5(1-7); PT 77% (278/361), 1(1-7); PTT 64% (238/361),1(1-7) and AST 40% (145/361) 3(1-6). Conjugated bilirubin and GGT were excluded from analysis because values for these were available in only 22% (80/361) and 20% (71/361) cases respectively.

Baseline patient characteristics are shown in Table 1, with comparison according to HIE grade and reception of hypothermia. The overall rate of culture-positive infection among the whole cohort was 2.8% (10/361) with no difference between the HIE grades or between hypothermia and normothermia groups. The results of histopathological placental examination were available for only 57 (15.7%) of the 361 infants, of which 18 (32%) showed evidence of chorioaminonitis and/or funisitis.[24]

Effects of HIE severity and therapeutic hypothermia on hepatic biomarkers

Table 2 presents the peak values of hepatic biomarkers measured in the first postnatal week, and the proportions of infants having a raised value for each biomarker according to HIE grade. The peak values of the hepatic biomarkers of injury including ALT and AST increased

with severity of HIE grade ($p<0.001$). Similarly, higher proportions of infants were affected with abnormally elevated ALT and AST concentrations with increasing HIE severity.

The biomarkers reflecting hepatic synthetic function, namely albumin and PT, differed according to HIE grade: infants with more severe HIE had significantly lower nadir albumin concentrations and lower peak total bilirubin concentrations (both $p<0.0001$), and a longer PT ($p<0.0001$), Table 2. Proportions of infants affected by an abnormally low plasma albumin value and a prolonged PT were also higher with increasing HIE severity (Table 2, figure 1).

Table 3 shows the results of univariate analysis according to reception of hypothermia treatment. Comparison between the hypothermia-treated and normothermia groups showed lower nadir albumin concentrations and longer PT and PTT times with hypothermia, but no differences for the other hepatic biomarkers. Sub-grouping according to grade of HIE showed significant differences associated with hypothermia reception for only a lower nadir albumin in grade 1 HIE and a longer PTT in grade 2 HIE (Online Resource Table 4). Univariate analysis limited to the sub-group of infants with moderate or severe encephalopathies (grades 2 and 3 HIE combined) showed only a longer PTT was associated with hypothermia therapy (Online Resource Table 4).

After adjusting for grade of HIE and birth weight in a multivariate regression analysis, only ALT and albumin were significantly affected by therapeutic hypothermia: infants in the hypothermia group had lower peak ALT ($p=0.025$) and a lower nadir plasma albumin ($p=0.049$) compared with the normothermia infants, and there were no differences between the hypothermia and normothermia-treated infants for any of the other biomarkers including AST, bilirubin, PT, and PTT (Table 5).

Effect of HIE severity and therapeutic hypothermia on peak CRP concentration

A raised CRP was present in 206/357 (57.7%) neonates during the first postnatal week (Table 2), with the peak occurring on postnatal day 4 overall. Proportions with a raised CRP increased with severity of HIE grade ($p<0.0001$). Considering only neonates with moderate or severe HIE (grades 2 and 3), it is noteworthy that the majority had a raised CRP within the first postnatal week (166/258; 64.3%), while only a small minority (8/246; 3.3%) had culture-positive sepsis (Table 1). CRP concentrations also peaked later in grades 2 and 3 HIE compared to grade 1 HIE ($p=0.0001$) (Table 2).

Univariate analysis showed that compared with HIE infants who did not receive hypothermia, the hypothermia-treated group had a higher peak CRP (15.4 versus 9.3 mg/L, $p=0.01$) and a higher proportion of infants with a raised CRP (62.6% versus 49.6%) (Table 3). After adjusting for HIE grade and birth weight, the multivariate regression showed no difference in peak CRP concentration between hypothermia and normothermia groups ($p=0.5$) however the time to peak was delayed in the hypothermia-treated group ($p<0.001$) (Table 5).

Figure 2 depicts changes in daily mean concentrations of peak CRP values over the first 7 days of life in normothermia and hypothermia groups with a delayed peak noted in infants who received hypothermia.

DISCUSSION:

With this study, we sought to determine the effect of hypoxic injury on surrogate biomarkers of hepatocellular integrity (ALT, AST) and hepatic synthetic function (albumin, PT) in neonates with HIE.[25] We believe this is the largest study to characterize markers of hepatic injury and function in setting of neonatal HIE [6-11] and, to our knowledge, the first to present baseline reference values for a range of hepatic biomarkers in the era of routine therapeutic hypothermia. Both hypoxic-ischemic neuronal and hepatic injury can occur secondary to perinatal asphyxia. We observed significant correlations between severity of HIE and values of several hepatic biochemical markers within the first 7 days after birth. More severe HIE was associated with greater elevation of hepatic enzymes (ALT, AST) and with abnormalities of markers of hepatic synthetic function (Albumin, PT).

Effect of severity of HIE on hepatic biochemical markers

Several smaller studies have reported an increase in some hepatic enzymes in infants with perinatal asphyxia and neonatal encephalopathy, including for AST and ALT.[3,6-10] Some have examined the correlation of hepatic biomarkers with severity of encephalopathy.[6,8-10] Of these, three reported significant correlation between hepatic enzymes and severity of neonatal encephalopathy,[6,8,10] while one study reported no correlation.[9] The inconsistency may be due to the relatively small numbers of infants studied, differing definitions of abnormal values of hepatic markers, and small cohorts making them relatively under powered for assessing correlations with HIE severity.

Changes in hepatic biomarkers with therapeutic hypothermia

Hypothermia limits neuronal injury in neonates with HIE,[26, 27] and improves neuro-developmental outcomes,[12-15] however effects of hypothermia on other organ systems are

less well studied. Vejchapipat et al. performed an experimental study using a rat model and reported that moderate hypothermia (30-33°C) ameliorates liver energy failure compared to controls after intestinal ischemic reperfusion injury.[28]. A meta-analysis of six randomized controlled trials which included 975 infants (316 of whom had hepatic dysfunction defined by using a higher threshold of AST >200 U/L and/or ALT >100 U/L), showed no significant hepato-protective effect of therapeutic hypothermia (relative risk 0.88 [95% CI: 0.74 to 1.05]).[29] However, the frequency and completeness of liver function testing in neonates in the included trials was unclear, and the use of a stricter definition of liver dysfunction may have decreased the sensitivity for detecting an effect. In our cohort, we observed inconsistent results for individual hepatic markers, with significantly lower peak ALT concentrations in the hypothermia group, but no difference for AST concentrations. This may possibly be due to the relatively lower number of babies with available AST samples. Nevertheless, ALT is considered to be a more specific marker for hepatic injury than AST which can be elevated due to other non-hepatic causes.[7,25] The biomarkers of hepatic function again showed varying results with a marginally lower albumin in the hypothermia group ($p=0.049$), but no difference in PT. The latter result is consistent with the meta-analysis of randomized controlled studies of therapeutic hypothermia which found no difference in coagulopathy between the hypothermia and control groups.[29]

Effect of severity of HIE and hypothermia on CRP responses

Several studies have examined CRP concentrations in the setting of HIE.[17-22] Shang et al. found a higher CRP concentration correlated with increasing clinical HIE severity in 74 infants.[18] Our study in a much larger cohort confirms that peak CRP concentrations and also proportions affected by a raised CRP both correlate strongly with HIE severity. Indeed a raised CRP appears to be an expected finding during the first postnatal week in neonates admitted with moderate or severe HIE; in our cohort this was nearly always in the absence of

infection because the rate of culture-positive infection was only 2.8% overall. This discrepancy may have clinical implications because it may help influence a more judicious use of antibiotics in infants admitted with HIE, particularly in those with negative cultures, and perhaps a higher threshold for performing repeated full infection screens later in the first week in the presence of a raised CRP despite invariable initial antibiotic treatment. Despite the low rate of proven sepsis, we found a high rate of chorioamnionitis (32%) for those infants whose placentas had been submitted for examination, highlighting the importance of formal routine placental examination in infants admitted after perinatal asphyxia.[24]

Hypothermia is known to modulate leucocyte and immune responses with altered and delayed expression of inflammatory mediators and cytokines including IL-6. [19-21]. CRP is an acute phase reactant protein produced in the liver in response to the pro-inflammatory cytokine IL-6.[30] Perrone et al. and Chakkarapani et al. compared neonates with encephalopathy who received therapeutic hypothermia with controls who were not treated with hypothermia and also reported a delayed CRP response in hypothermia-treated infants.[20, 21] Okumus et al. recently showed that CRP responses were altered with therapeutic hypothermia with significantly higher levels of CRP, which peaked at day 4 of life compared to a normothermia group which showed no variation in CRP with time.[22] While we did not find any difference in peak CRP levels between our hypothermia-treated infants and those managed at normal temperature, we nevertheless also found a delay in peak CRP responses with therapeutic hypothermia, in line with these previous studies.[20-21]

Our study has a few limitations. This was a retrospective study and hence data were not available for all desired variables. Not all NICUs measured all hepatic biomarkers routinely and consistently, therefore we needed to exclude the biomarkers GGT and conjugated bilirubin where a high proportion of biomarkers were unmeasured. Furthermore, we recognize that not all measured hepatic enzymes and biomarkers are wholly specific for

the liver, for example AST and PT can be elevated due to non hepatic causes. Similarly CRP is commonly elevated in infection and in other inflammatory conditions, although rates of culture-positive sepsis were very low in our cohort. Therapeutic hypothermia was introduced at different intervals in our participating centers during the study period and the normothermia group included infants with moderate and severe HIE, who may have qualified for therapeutic hypothermia before it became standard care. To address this limitation, we performed multiple regression analysis to adjust for effect of severity of HIE on hepatic biomarkers whilst assessing for influence of therapeutic hypothermia. Our analysis of biomarkers was confined to samples obtained in the first postnatal week, and more longitudinal variation in these biomarkers remains unknown. Strengths of our study are that it presents data on hepatic markers associated with HIE in the largest cohort to date, and that it provides preliminary reference ranges for a number of hepatic biomarkers in encephalopathic infants, most of whom received therapeutic hypothermia.

Conclusion:

In our retrospective study of a large cohort of infants with HIE, we have observed a significant association between the clinical grade of HIE and several markers of liver metabolism and function. Therapeutic hypothermia was associated with delayed CRP responses and with lower ALT and albumin concentrations. More studies will be required to prove a definitive effect of hypothermia on limiting hepatic injury ~~and preserving hepatic function~~. However, as hypothermia is now standard treatment in moderate-severe HIE, future prospective controlled studies will not be possible in human infants and the best inferences may therefore need to come from animal models.

Compliance with Ethical Standards:

Conflict of interest statement: There are no competing interests and no conflicts of interests to declare in relation to this work.

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Ethical approval: This article does not contain any studies with human participants or animals performed by any of the authors and informed consent was not required for the purpose of the study. This study was done with the approval of the National Research Ethics Service Committee East of England - Cambridge Central (REC reference: 11/EE/0349).

Author contributions :

Paul Clarke conceived the idea for this study. Hemananda Muniraman and Paul Clarke designed the study protocol, drafted the data collection form, and obtained the ethics approval. Hemananda Muniraman, Paul Clarke, Sunil Sanka, Danielle Gardner, Anna Paweletz, Anitha Vayalakkad, Ying Hui Chee, Clare Clifford, and Vidheya Venkatesh collected the data from the four centers. Data were analyzed by Jane Skinner, Hemananda Muniraman and Paul Clarke. Anna Curley, Suresh Victor, Mark Turner, and Paul Clarke obtained local approvals for their centers, verified data queries, and provided intellectual input. Hemananda Muniraman and Paul Clarke wrote the first manuscript draft. All authors contributed to manuscript drafting and approve the final version. **Paul Clarke is guarantor.**

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Figure legends:

Table 1. Baseline characteristics of the 361 infants admitted with hypoxic-ischemic encephalopathy with subdivision according to encephalopathy grade and reception of hypothermia

Table 2. Concentrations of hepatic enzymes and hepatic biomarkers measured in the first postnatal week, with subdivision according to grade of hypoxic ischemic encephalopathy

Table 3. Concentrations of hepatic enzymes and hepatic biomarkers measured in the first postnatal week in normothermia and therapeutic hypothermia groups.

Table 4. (Online Resource) Concentrations of hepatic enzymes and hepatic biomarkers measured in the first postnatal week in normothermia and therapeutic hypothermia groups based on grade of HIE.

Table 5. Regression coefficients of log (biomarkers) with therapeutic hypothermia

Figure 1. Hepatic biomarkers and grades of HIE: The biomarkers are reported in median with interquartile ranges. All infants with at least one measurement available were included.

Figure 2. Peak CRP levels (means with standard error) in the first 7 days of life in therapeutic hypothermia and normothermia groups. All infants with at least one CRP measurement available were included.